

PHOTOREACTIVE COMPOUND SPECIFICALLY BINDING
TO CALCIUM BINDING PROTEINS

Field of the Invention

5 The present invention relates to a photoreactive reagent that binds specifically to Ca^{2+} -binding proteins, links to them covalently after photo-activation, and thus labels them and enables their characterization and purification. The invention also provides methods for the preparation of affinity chromatography matrix and protein biosensors comprising said
10 reagent, and relates to therapeutic and diagnostic uses of said reagent and biosensors.

Background of the Invention

Calcium (Ca^{2+}) is a ubiquitous intracellular signal transducer, responsible for
15 controlling numerous cellular processes. It regulates muscle contraction, neurotransmitter release, hormone secretion, cell motility, cell mitosis, and gene expression. Ca^{2+} -signaling is employed throughout the life of the organism, beginning with a surge of Ca^{2+} at fertilization and ending with the induction of apoptosis, thereby, at cell death. Calcium is deeply involved in
20 the cell proliferation. Several drugs that block Ca^{2+} entry into the cell, retard the cell growth in human melanoma, lung and colon carcinoma, and prostate cancer [See, e.g., Haverstick, D.M. et al.: Cancer Res. 60 (2000) 1002-8].

There are many proteins involved in the compound role of calcium in the organism having high-affinity or low-affinity Ca^{2+} -binding sites. Many of said
25 proteins remain unknown or insufficiently characterized. Despite advances in defining Ca^{2+} -dependent activity, considerable experimental difficulties still remain in localizing the protein Ca^{2+} -binding sites [Hadad N. et al.: J. Biol. Chem. 269 (1994) 26864-9]. Fundamental to the understanding of normal and abnormal calcium signaling is the knowledge of the proteins
30 involved in this process. It is therefore an object of this invention to provide a compound binding specifically to Ca^{2+} -binding proteins.

It is an object of this invention to provide a photoreactive azido-ruthenium (AzRu) based probe which specifically binds to Ca^{2+} -binding proteins, and inhibits their activity.

- 5 It is another object of this invention to provide an AzRu-based probe which specifically and covalently binds to Ca^{2+} -binding sites of Ca^{2+} -binding proteins, thereby allowing to localize and to characterize such sites.

- 10 It is another object of this invention to provide the use of said AzRu-based compound in producing affinity chromatography matrices binding specifically Ca^{2+} -binding proteins, and allowing their purification.

- 15 It is still another object of this invention to provide the use of said AzRu-based compound in producing biosensors binding specifically to Ca^{2+} -binding proteins, and allowing their characterization.

It is further an object of this invention to provide diagnostic and therapeutic uses of azido-ruthenium compound associated with defects in the function of Ca^{2+} -binding proteins.

20

Summary of the Invention

- This invention provides a new photoreactive azido-ruthenium compound, which exhibits a specific interaction with Ca^{2+} -binding proteins. The reagent, azido-ruthenium of this invention (AzRu), was found to specifically inhibit
25 activities of various Ca^{2+} -transport mediating proteins, Ca^{2+} -regulatory proteins, and Ca^{2+} -dependent enzymes. When photo-activated, the reagent binds covalently to the calcium binding site. Azido-ruthenium according to this invention can be synthesized from radiolabeled reagents, which enables to label binding sites in Ca^{2+} -binding proteins, and to characterize these
30 important proteins. The compound comprises ruthenium and azide in the molar ratio of 2:1. Said AzRu compound binds covalently after photo-activation by UV irradiation to the Ca^{2+} -binding site of said protein. The

binding is specific for Ca^{2+} -binding proteins. In a preferred embodiment of this invention, the compound contains a radioactive isotope, preferably ^{103}Ru , and can label radioactively the binding sites in said Ca^{2+} -binding proteins. The compound of this invention can bind to Ca^{2+} -binding proteins, and
5 inhibit their activity. Among the proteins which can bind the compound of this invention are Ca^{2+} -binding proteins selected from proteins involved in signal transduction, muscle contraction, neurotransmitter release, hormone secretion, cell motility, apoptosis, fertilization, cell proliferation, cell mitosis, gene expression: where some of these proteins are associated with mediating
10 Ca^{2+} transport, such as Ca^{2+} -pump, the mitochondrial uniporter, channel protein VDAC (voltage dependent anion channel), Ca^{2+} release channel/ryanodine receptor, proteins involved in Ca^{2+} uptake in mitochondria, and the Ca^{2+} pump catalyzing the transport of Ca^{2+} across the sarcoplasmic reticulum membrane. When the compound of this invention is
15 photo-activated after its binding to a protein by UV-irradiation, it binds covalently and thus inhibition of the activity of said protein increases several-fold, compared to the inhibition without photo-activation.

The invention also relates to the method of purifying, identifying, and
20 characterizing Ca^{2+} -binding proteins by employing AzRu compound containing ruthenium and azide in molecular ratio of 2:1. When studying function and structure of Ca^{2+} -binding proteins, the method of this invention may be combined with methods known in the art, such as SDS-PAGE, autoradiography, MALDI-TOF analysis, LC-MS/MS, protein sequencing, and
25 a sequence homology search. In a preferred embodiment, this invention provides new affinity chromatography technique, comprising binding AzRu to particles of porous polymer that are packed in a column, wherein Ca^{2+} -binding proteins are retained in said column, while other proteins pass. Said retained proteins are released by calcium ions. Said particles may comprise
30 agarose, cellulose, or other matrix. Said method of this invention may comprise purifying Ca^{2+} -binding proteins, identifying unknown Ca^{2+} -binding proteins, inhibiting Ca^{2+} -binding proteins, and labeling Ca^{2+} -binding proteins.

Said labeling may be a radioactive labeling. In a preferred embodiment, the method of this invention comprises the preparation of an affinity resin. In another preferred embodiment, the method according to this invention comprises the preparation of a bio-sensor chip.

5

The invention provides a process for preparing a photoreactive azido-ruthenium reagent that binds to Ca^{2+} -binding proteins, which comprises i) reacting in dark of sodium azide with ruthenium (III) chloride in the presence of HCl, ii) applying the reaction mixture of the previous step onto a chromatographic column, e.g. cation-exchange or hydrophobic interaction column, iii) collecting the fractions containing AzRu, wherein such fractions may be identified, e.g., according to the absorbance at 290 nm, iv) drying the collected fractions, and optionally dissolving said dried fractions and applying them again on a chromatographic column, and drying obtained AzRu reagent. The reagent may eventually be crystallized from methanol. In the process of this invention, HCl has preferably the concentration in the range from 0.5 mol/l to 2 mol/l, and sodium azide and ruthenium chloride react at about 100°C for about 2 to 4 hrs. This invention further provides a compound of empirical formula $\text{Ru}_2\text{N}_3\text{Cl}_{5.5}\text{H}_2\text{O}$, and solvates thereof, e.g. solvates further comprising H_2O and HCl.

This invention is also directed to the use of AzRu compound containing ruthenium and azide in a molar ratio of 2:1 in diagnosing disorders associated with Ca^{2+} -binding proteins, and in preparing a medicament for treating said disorder. The invention also relates to a pharmaceutical composition containing said AzRu compound, or a solvate thereof, and possibly a carrier, stabilizer, adjuvant, diluent, or excipient. Said AzRu compound, or a composition containing it, may be used in the preparation of a medicament for treating or preventing a disorder associated with a defect in the function of a Ca^{2+} -binding protein, wherein said preventing and treating comprise the inhibition of Ca^{2+} -binding proteins by said AzRu compound.

Brief Description of the Drawings

The above and other characteristics and advantages of the invention will be more readily apparent through the following examples, and with reference to the appended drawings, wherein:

- 5 Fig. 1. shows the purification of AzRu on Sephadex LH-20 column;
Fig. 2. shows the characterization of AzRu by TLC;
Fig. 3. shows UV spectra of AzRu, ruthenium red, RuCl_3 , and NaN_3 ;
Fig. 4. shows IR spectrum of AzRu;
Fig. 5. shows the purification of radioactive AzRu on Sephadex LH-20
10 column;
Fig. 6. A and B demonstrate the inhibition by AzRu of the Ca^{2+} -pump activity in skeletal muscle membranes;
Fig. 7. A to C show the effect of AzRu on calcium uptake and electron transport in isolated mitochondria;
15 Fig. 8. shows the inhibition of ryanodine binding to its receptor by AzRu;
Fig. 9. shows the inhibition of VDAC channel activity by AzRu;
Fig. 10. A and B show the identification of Ca^{2+} -binding proteins on SDS-PAGE by using $[^{103}\text{Ru}]\text{AzRu}$;
Fig. 11. illustrates the use of AzRu in affinity chromatography;
20 Fig. 12. shows SDS-PAGE of Ca^{2+} -binding proteins isolated by AzRu-affinity chromatography; and
Fig. 13. A and B show the protection of U-937 cells against STS-induced apoptotic cell death by AzRu.

Detailed Description of the Invention

This invention provides a new azido-ruthenium, which is photoreactive, and exhibits a specific interaction with Ca^{2+} -binding proteins. Synthesis of three azido-ruthenium compounds were reported [Seok W.K. et al.: J.Organometallic Chem. 559 (1998) 65-71; Siebald H.G.L. et al.: Polyhedron
30 15 (1996) 4221-5; Vrestal J. et al.: Collection Czechoslov. Chem. Commun. 25 (1960) 2155-60]. Effects of these non-photoreactive reagents on biological

activities were not described. Another known ruthenium reagents, ruthenium red and Ru360 are not photoreactive. The preferred reagent of this invention can be prepared by reacting sodium azide with ruthenium chloride (III) and purifying the product on a chromatographic column, e.g. cation-exchanger or hydrophobic. Chromatography purification methods, 5 useful for this purpose comprise, for example, CM-cellulose or Sephadex LH-20 columns. IR spectrum of the novel azido-ruthenium (AzRu) product shows the characteristic absorption of the bound azido group at 2072 cm^{-1} . It was surprisingly found that the azido-ruthenium compound of this invention 10 interacts with Ca^{2+} -binding proteins and inhibits their activity. When photo-activated, by UV-irradiation, the reagent binds covalently to the Ca^{2+} -binding sites. The effect of the reagent of this invention on activities of various proteins, including channels, receptors and enzymes, were tested, and it was found by us that the interaction is specific for the Ca^{2+} -binding proteins 15 (Table 1). Azido-ruthenium according to this invention can be synthesized from radiolabeled reagents, thus enabling labeling of binding sites in Ca^{2+} -binding proteins. Such labeling enables discovering still unknown proteins, explaining still unclear aspects of regulations and signal transduction pathways depending on calcium ions, separating and characterizing the 20 involved peptides and proteins, as well as determining their structure and function.

In one embodiment, to identify Ca^{2+} -binding proteins, proteins labeled by radiolabeled reagents are separated by SDS-PAGE, identified by 25 autoradiography, cut from the gel and exposed to cleavage conditions. The degradation products are separated by a second SDS-PAGE, and the Coomassie stained labeled bands are sequenced to identify the Ca^{2+} -binding sites. The labeled protein bands can be cut from electrophoresis gels, and subjected to MALDI-TOF analysis or LC-MS/MS, and the identity of the 30 protein can be determined by a sequence homology search.

Above mentioned approach can be used, for example, for identifying mitochondrial systems for transporting Ca^{2+} , like ruthenium red-sensitive uniporter, Na^+ or H^+ dependent efflux mechanisms, and the rapid mode of uptake transporter.

5

Our results show that azido-ruthenium of this invention specifically inhibits the activity of proteins which mediate Ca^{2+} transport such as the muscle Ca^{2+} -pump, the mitochondrial uniporter, and the channel protein VDAC. An AzRu compound according to this invention also inhibits the activity of
10 proteins possessing regulatory Ca^{2+} -binding sites such as the muscle Ca^{2+} -release channel/ryanodine receptor and calmodulin. In contrast to that, azido-ruthenium has no significant effect on Ca^{2+} -independent enzymatic reactions.

15 Using radioactive AzRu, Ca^{2+} -binding proteins can be identified, purified and their functions can be established, e.g., autoradiography helps to localize suspected proteins. In a preferred embodiment, the radioisotope used for the synthesis of AzRu is ^{108}Ru .

20 In another preferred embodiment, the AzRu reagent of this invention is used for separation of Ca^{2+} -binding proteins by affinity chromatography, wherein the AzRu reagent is covalently attached to an inert support filled in chromatographic column. The support can be, for example, a porous polymer, such as agarose, cellulose, or dextrane, etc. A protein mixture is loaded into
25 the column, Ca^{2+} -binding proteins are reversibly retained while other proteins pass. The retained proteins may be released, for example, by a calcium containing buffer.

In another embodiment of this invention, AzRu is coupled to polysaccharides,
30 and is used for construction of unique and novel bio-sensor chips for isolation and identification of Ca^{2+} -binding proteins. In a preferred embodiment, this invention enables to identify proteins that have affinity for Ca^{2+} -binding

proteins, i.e. proteins that are not necessarily calcium binding themselves, but interact with Ca^{2+} -binding proteins, forming elements of the signal transduction pathways, often elements that are difficult to detect and identify. In a preferred embodiment of this invention, surface plasmon resonance (SPR) is used for detecting intermolecular interactions. The chip of this invention can be used, for example, for identifying new Ca^{2+} -binding proteins, or sites of interaction; binding affinity and kinetics, as well as epitope map can be also obtained.

Based on the above findings, the present invention is also directed to a pharmaceutical composition for use in inhibiting calcium dependent proteins involved in various disorders associated with Ca^{2+} -binding proteins and defects in these proteins.

The invention provides a process for preparing an azido-ruthenium compound, having the ratio Ru:N_3 of 2:1, that specifically binds to, and inhibits, Ca^{2+} -binding proteins, comprising the steps of:

i) reacting in dark of sodium azide with ruthenium (III) chloride in the presence of HCl, ii) applying the reaction mixture of the previous step onto a chromatographic column, e.g. cation-exchanger or hydrophobic, iii) collecting the fractions which contain the required product, possibly identifying such fractions by measuring absorbance; and optionally iv) steps of drying the collected fractions, redissolving them, rechromatographing them, and drying said compound from methanol, eventually crystallizing it. The process of this invention provides an AzRu product that migrates as a single spot with R_f being about 0.9 during TLC on cellulose F plates, using 0.16 M ammonium formate, pH 8.5 and 20% methanol, said product having an absorbance maximum at about 290 nm, and its absorbance is preferably about from 10,000 to 20,000 at a concentration of 1.0 M in a water solution, usually said absorbance at 290 is about 15,000.

Thus, this invention provides a novel tool to identify, purify and characterize Ca^{2+} -binding proteins, and to sequence the Ca^{2+} -binding sites. In addition, the requirement for Ca^{2+} in a protein reaction or in the regulation of its activity can be tested. The invention may be useful as a pharmaceutical agent to monitor the involvement of Ca^{2+} in the activity or regulation of proteins. The invention can be used as well for the detection of defected Ca^{2+} -binding proteins in certain diseases and disorders.

The invention will be further described and illustrated in the following examples.

Examples

Materials and general methods

Chemicals

Aldolase, alkaline phosphatase, ATP, bovine liver catalase, choline oxidase, CM-cellulose, n-decane, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, Hepes, yeast hexokinase, lactate dehydrogenase, leupeptin, luciferase, mannitol, horseradish peroxidase, PMSF, pyruvate kinase, soybean asolectin, sucrose, Tris and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). ^{45}Ca , ^{103}Ru and ^3H ryanodine were purchased from NEN® Life Science Products, Inc. (Boston, USA). Unlabelled ryanodine was obtained from Calbiochem. Sephadex LH-20 was purchased from Amersham Biosciences. n-Octyl- β -D-glucopyranoside (β -OG) was obtained from Bachem AG (Germany). Lauryl-(dimethyl)-amineoxide (LDAO) and ruthenium red (98% pure) were obtained from Fluka (Chemie, GmbH). Ruthenium chloride was purchased from Aldrich. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories (Hercules, CA) and Celite from Merck. Synthetic firefly D-luciferin reagent was purchased from Biosynth AG.

Preparations

Ru360 was synthesized according to Ying [Ying et al.: Biochemistry 30 (1991) 4949-52].

5 Sarcoplasmic reticulum (SR) membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito [Saito et al.: J.Cell Biol. 99 (1984) 875-85]. Mitochondria were isolated from rat liver as described by us previously [Gincel D. et al.: Biochem J. 358 (2001) 147-55]. VDAC was purified by a method developed in our laboratory, using columns of
10 hydroxyapatite and reactive red agarose [Gincel D. et al.: J. Bioenerget. Biomembr. 32 (2000) 571-83].

Calsequestrin was isolated from rabbit skeletal muscle sarcoplasmic reticulum membranes as described by Cala S.E. et al. [J. Biol. Chem. 258
15 (1983) 11932-6]. Calmodulin was isolated from sheep brain as described by Gopalakrishna R. et al. [Biochem. Biophys. Res. Commun. 104 (2) (1982) 830-6]. Troponin was isolated from rabbit skeletal muscle as described by Potter J.D. [Methods in Enzymol. 85 (1982) 241-65].

20 Assays

Protein concentrations were determined by the standard Lowry procedure [Lowry O.H. et al: J. Biol. Chem 193 (1951) 224-265]

For the determination of [³H]ryanodine binding, SR membranes were
25 incubated for 20-60 min at 37°C in a standard binding solution containing 1M NaCl, 20 mM MOPS (pH 7.4), 50 µM free Ca²⁺ and 20 nM [³H]ryanodine. Unbound ryanodine was separated from protein-bound ryanodine by vacuum filtration of the sample through nitrocellulose filters (0.45 µm), followed by two washes with 4 ml ice-cold buffer containing 0.2 M NaCl, 10 mM MOPS
30 (pH 7.4) and 50 µM CaCl₂. The retained radioactivity in the dried filters was determined by liquid scintillation counting. Specific binding of [³H]ryanodine

was defined as the difference between the binding in the presence of 20 nM [³H]ryanodine and in the presence of 20 μM unlabeled ryanodine.

- Ca²⁺-accumulation in freshly prepared mitochondria (0.5 mg/ml) was assayed
5 for 1 to 20 minutes at 30°C in the presence of 225 mM mannitol, 75 mM
sucrose, 120 μM CaCl₂ (containing 3x10⁴ cpm/nmol ⁴⁵Ca²⁺), 5 mM
HEPES/KOH pH 7.0, and 5 mM succinate with 0.1 mM Pi, or 4 mM MgCl₂
with 3 mM ATP. The Ca²⁺-uptake was terminated by rapid Millipore
filtration, followed by a wash with 5 ml of 0.15 M KCl. The Ca²⁺-
10 accumulation in SR was determined as described previously [Shoshan-
Barmatz V. & Shaineberg A.: Biochim. Biophys. Acta 1065 (1991) 82-8].
Electron transport from succinate to cytochrome c was described in above
cited article [Gincel D. et al.: J. Bioenerget. Biomembr. 32 (2000) 571-83].
- 15 Reconstitution of purified VDAC into planar lipid bilayer (PLB), single
channel current recording, and data analysis were carried out as described in
above mentioned publication.

Example 1

20 Azido-ruthenium (AzRu) synthesis

- All steps were carried out in the dark. RuCl₃ (29.8 mg, 0.144 mmol) and
NaN₃ (6.24 mg, 0.096 mmol) were dissolved in 1N HCl (2 ml), silted and
incubated at 100°C for 3 hrs. The sample was then applied to a Sephadex LH-
20 column (1.5 cm x 44 cm) pre-equilibrated with water (Fig. 1). Free Ru³⁺,
25 but not the product, remained bound to the column matrix. The absorbance
at 290 was measured and the peak was collected, lyophilized and analyzed by
TLC on cellulose F plates using 0.16 M ammonium formate, pH 8.5 and 20%
methanol as a developer. The product migrated as a single spot with R_f = 0.9
(Fig. 2). The product is soluble in water, DMF and DMSO, less soluble in
30 methanol, and insoluble in ethanol, ether, chloroform, ethyl acetate, n-
butanol, and isopropyl alcohol. The purified product of azido-ruthenium has a
maximal absorbance at 290 nm, the absorbance of a solution of 1.0 M being

about 15,000. The spectrum of the product is different from that of the substrates and of ruthenium red (RuR), a known inhibitor of calcium dependent activity, which is not photoreactive (Fig. 3). Infrared spectrum of the product indicates the presence of a specific peak representing a bound
5 azido group (Fig. 4). For further purification, the dried product was dissolved in water and subjected to a second chromatography on Sephadex LH-20. The elution of radioactive product [^{103}Ru]AzRu is shown in Fig. 5.

Example 2

10 Purification and characterization of AzRu

AzRu was prepared as in Example 1, the product eluting as the second peak (Fig. 1), having the absorption maximum at 290 nm, was dried. The elemental analysis of the compound was carried out by SGS Cervac Wolff (France) and the composition was determined in the Department of
15 Chemistry of Ben Gurion University, Israel, to provide the empiric formula of the product: $\text{Ru}_2\text{N}_3\text{Cl}_{5.5}\text{H}_2\text{O}$ (H_2O , HCl).

Example 3

Inhibition of Ca^{2+} -pump activity

20 The effect of azido-ruthenium on Ca^{2+} -dependent proteins and its photoactivation was demonstrated by the inhibition of the Ca^{2+} -pump activity involved in muscle relaxation as shown in Fig. 6. The preparation of SR membranes from skeletal muscle, and the measurement of Ca^{2+} -accumulation were carried out as described above. The sensitivity of the
25 Ca^{2+} -pump activity to azido-ruthenium was increased more than two-fold by photoactivation, demonstrating that azido-ruthenium of this invention is a photoreactive compound. Figure 6 shows that the inhibition is dependent on time of UV irradiation, as well as on the concentration of the reagent. UV irradiation increases the inhibitory effect of the compound on Ca^{2+} -
30 accumulation by SR membranes, confirming the photoactivation, and suggesting irreversible binding of the reagent to the protein.

Example 4

Specific inhibition of Ca^{2+} transport in mitochondria

The effect of AzRu on mitochondrial process of electron transport from the donor succinate to the acceptor cytochrome c was characterized as described
5 above. The effect of azido-ruthenium on the Ca^{2+} -uptake, and on the electron transport in mitochondria is shown in Fig. 7. Although said electron transfer involves several dozens electron transfer carriers, this reaction was not affected by azido-ruthenium of this invention. On the other hand, Ca^{2+} transport, which is carried out by a yet unidentified uniporter protein, was
10 inhibited by azido-ruthenium, demonstrating the specificity of azido-ruthenium of this invention.

Example 5

Inhibition of ryanodine binding to SR membranes by AzRu

15 The effect of azido-ruthenium on the binding of the toxic alkaloid ryanodine to its receptor, known as the Ca^{2+} -release channel that possesses regulatory Ca^{2+} -binding sites, was measured as described above. Azido-ruthenium of this invention strongly inhibited the binding of ryanodine to SR membranes, as demonstrated in Fig. 8, in which a comparison is also shown with Ru360.

20

Example 6

VDAC channel closure

VDAC is a channel protein which transports Ca^{2+} across the mitochondrial outer membrane. It possesses Ca^{2+} -binding sites [Gincel D. et al.: Biochem J.
25 358 (2001) 147-55] and thus is involved in regulation of its own activity. Azido-ruthenium inhibited the channel activity of VDAC. Purified VDAC was reconstituted into a planar lipid bilayer as multichannels. The channel activity was measured as the ions passed the current across the bilayer in response to a voltage gradient of 10 mV, as a function of time. In the absence
30 of AzRu, VDAC is stable in a long-lived, fully opened state and remains open for up to 2 hours of recording. However, addition of AzRu to the same channel induced VDAC closure, in a time-dependent manner. Ca^{2+} , in the

presence of 1 M NaCl, prevented the inhibitory effect of AzRu on VDAC activity. As has been shown for RuR [Gincel D. et al.: Biochem J. 358 (2001) 147-55], chelation of Ca^{2+} with EGTA re-established AzRu inhibition, suggesting specific interaction of AzRu with VDAC Ca^{2+} -binding sites. AzRu also decreased VDAC conductance in multi-channel experiments at all voltages tested and stabilized VDAC conductance at a constant low level regardless of the voltage gradient applied. Similar results have been obtained with RuR and Ru360.

Example 7

10 Specific inhibition of Ca^{2+} -dependent reactions by AzRu

The specificity of the binding of AzRu to Ca^{2+} -binding proteins is demonstrated by testing its effects on the activity of different enzymes. The results, summarized in Table 1, indicate that the activities of Ca^{2+} -independent proteins such as glutamate dehydrogenase, lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, choline oxidase, catalase, lysozyme, aldolase, alkaline phosphatase, as well as electron transfer in mitochondria involving over two dozen polypeptides, were only slightly inhibited by the reagent (less than 10% at 100 μM). In contrast, the activities of Ca^{2+} -dependent proteins were strongly inhibited by azido-ruthenium (up to 90% at $\leq 20 \mu\text{M}$). The inhibition was observed for various proteins with different activities. Such proteins include, for example, proteins which catalyze the transport of Ca^{2+} across the membrane by distinct mechanisms, all of which involve Ca^{2+} -binding to the protein, like the Ca^{2+} -pump that transports Ca^{2+} at the expense of ATP hydrolysis, and the uniporter protein which transports Ca^{2+} accumulated in the mitochondrial matrix as a result of the membrane potential established by the electron transport chain. Another inhibited activity is that of the ryanodine receptor, Ca^{2+} -release channel, which possesses regulatory Ca^{2+} -binding sites that control its channel activity and its ability to bind the toxic alkaloid ryanodine. The results also indicate that the activities of Mg^{2+} -dependent proteins, such as yeast and brain hexokinase, pyruvate kinase, luciferase and $(\text{Na}^+/\text{K}^+)\text{ATPase}$, and the Zn^{2+} -

sensitive KCNK0 channel were not affected or were weakly inhibited at high AzRu concentrations (~30% at 100 μ M).

Table 1 Effect of AzRu on Ca^{2+} - or Mg^{2+} - dependent or -independent or Zn^{2+} sensitive proteins, as described in Example 7. Protein activity was assayed in the absence and the presence of different concentrations of AzRu, and the degree of inhibition by 20 or 100 μ M of AzRu is presented. The results are the mean \pm SEM of 3 to 6 experiments.

Activity assayed	Activity (% of control)		Divalent cation involved
	AzRu (20 μ M)	AzRu (100 μ M)	
1. Ca^{2+} uptake in SR (SERCA)	13 \pm 6		Ca^{2+}
2. Ca^{2+} uptake in mitochondria	11 \pm 4		
3. Ryanodine binding by SRRyR	18 \pm 6		
4. VDAC conductance	19 \pm 5		
5. Peroxidase	39 \pm 4		
6. CaMKII-dependent GAPDH	6 \pm 2		
7. Ca^{2+} -dependent mitochondrial swelling (c)	0		
8. Yeast hexokinase	77 \pm 5	64 \pm 4	Mg^{2+}
9. Brain hexokinase	73 \pm 7	61 \pm 3	
10. Pyruvate kinase	97 \pm 7	81 \pm 6	
11. Luciferase	97 \pm 1	67 \pm 3	
12. (Na^+/K^+)ATPase	92 \pm 5	71 \pm 4	
13. K^+ channel (KCKN0)	100 \pm 0.3		Zn^{2+}
14. Glutamate dehydrogenase (b)		101 \pm 5	None
15. Lactate dehydrogenase		91 \pm 4	
16. G-6-P dehydrogenase		101 \pm 7	
17. GAP dehydrogenase		102 \pm 5	
18. Alkaline phosphatase		86 \pm 5	
19. Succinate-cytochrome c oxidoreductase		95 \pm 5	
20. Lysozyme		101 \pm 3	
21. Aldolase		78 \pm 7	
22. Catalase		118 \pm 8	
23. Choline oxidase		85 \pm 3	

(a) PTP opening was completely inhibited at this concentration of AzRu.

(b) The enzyme possesses Ca^{2+} -binding site(s) that are not involved in its activity.

Example 8

Identification of unknown Ca^{2+} -binding proteins

By using radiolabeled $[^{103}\text{Ru}]$ azido-ruthenium, it is possible to identify Ca^{2+} -binding proteins in a biological sample such as whole cells, isolated mitochondria, ER, or other protein containing fractions. UV irradiation of SR or mitochondria in the presence of $[^{103}\text{Ru}]\text{AzRu}$ resulted in its covalent binding to several proteins, as revealed by SDS-PAGE followed by Coomassie staining and autoradiography. As expected for the photoreactive $[^{103}\text{Ru}]\text{AzRu}$, the labeling of the proteins was significantly increased upon UV irradiation, indicating the formation of a stable covalent bond. In skeletal muscle SR incubated with 0.5 nmol $[^{103}\text{Ru}]\text{AzRu}$ /mg protein (0.5 μM) with or without UV irradiation, nine out of about thirty proteins stained by Coomassie were labeled with $[^{103}\text{Ru}]\text{AzRu}$. Among the labeled proteins are known Ca^{2+} -binding proteins such as RyR, myosin, Ca^{2+} -ATPase and calsequestrin, but there were also two unidentified proteins of 37 and 25 kDa (Fig. 10 A). Similarly, in an isolated mitochondrial membranal fraction from which soluble proteins were extracted, photoactivated $[^{103}\text{Ru}]\text{AzRu}$ labeled seven out of about twenty seven proteins stained by Coomassie (Fig. 10 B). Weak $[^{103}\text{Ru}]\text{AzRu}$ labeling of the proteins was observed with no UV irradiation indicating a very stable interaction, which is not surprising when considering the reported labeling of mitochondrial proteins with the non-photoreactive reagent $[^{103}\text{Ru}]\text{Ru360}$ [Zazueta C. et al.: J. Bioenerg. Biomembr. 30 (1998) 489-98].

Example 9

Affinity chromatography (Afch)

The specific reversible interaction between a Ca^{2+} -binding protein and azido-ruthenium can be exploited in separation of Ca^{2+} -binding proteins by affinity chromatography (Fig.11). Azido-ruthenium of this invention can be coupled to an inert support such as agarose, polyacrylamide or polystyrene by photoactivation, for example cellulose or Sepharose^R beads can be used for said covalent attachment. From the proteins applied to the column, only

Ca²⁺-binding proteins interact with the ruthenium bound in the column, and are retained. The retained proteins can be eluted with a Ca²⁺-containing buffer. Proteins eluted with Ca²⁺ represent Ca²⁺-binding proteins. The proteins can be sequenced and the sequences can be used for the sequence
5 homology search and identification of the proteins.

Example 10

Purification of Ca²⁺-binding proteins using Afch on AzRu column.

AzRu, 4.5 mg, obtained as described in Examples 1 and 2, was incubated
10 with cellulose fibers (Sigma-Aldrich), 1.25 g, in the presence of 10 mM carbonate buffer, pH 7.5, irradiated by UV light for 7 times 2 minutes with short intervals between, and left overnight. The unbound AzRu was removed by washing with said buffer to provide AzRu-cellulose. The protein preparations were applied to the column, and, after washing with said buffer,
15 eluted from the column with said buffer further containing 2-20 mM CaCl₂.

Rat liver mitochondria, rabbit sarcoplasmic reticulum, rat brain extract, calsequestrin, calmodulin, and troponin were isolated as described above. In order to solubilize the rat liver mitochondrial membrane proteins,
20 mitochondria were treated with 3% Triton X-100, 2 % C₁₂E₉ or 3% Triton X-114. To some of the eluted proteins, either EGTA (2 mM) or CaCl₂ (2 mM) was added, and the samples were subjected to SDS-PAGE (Fig. 12). It can be seen that CaCl₂ shifts the electrophoretic mobility of the proteins, which was demonstrated for some known Ca²⁺-binding proteins [Gregersen H.J. et al.:
25 Adv. Exp. Med. Biol. 269 (1990) 89-92].

Among several dozens of proteins applied to the column, only few proteins were bound to the column and were eluted with CaCl₂. Some of the proteins that were eluted from the column with the CaCl₂ - containing buffer were
30 identified by MALDI-TOF analysis and are presented in Table 2. The results clearly indicate the specificity of the AzRu-cellulose column to Ca²⁺-binding proteins.

Table 2. Proteins eluted by affinity chromatography on AzRu column according to Example 10

Preparation applied	Protein MW (kDa)	Identification by MALDI TOF/ LC/MS	Metal binding
Rat liver mitochondria	69	Albumin precursor	+
	33	Malate dehydrogenase	+
	45	Aspartate aminotransferase	+
	28	Unknown	+
	36	Unknown	+
Rabbit skeletal muscle sarcoplasmic reticulum	26	Triosephosphate isomerase	+
	49	Enolase	+
	58	Unknown	+
Purified protein	Calsequestrin		+
	Calmodulin		+
	Troponin		+
Rat brain extract	Calbindin		+

Example 11

5 Development of novel AzRu-based bio-sensor chips

The specific interaction of AzRu with Ca^{2+} -binding proteins enables to develop a biosensor chip for real-time monitoring of macromolecular interactions with Ca^{2+} . It can be an optical biosensor using surface plasmon resonance (SPR). In principle, the SPR-based technique detects mass and has
 10 been designed for the study of biomolecular binding and therefore provides an invaluable tool for proteomics studies of the relationship between protein

structure and function. The AzRu-based bio-sensor chip is an optical biosensor that contains AzRu interacting specifically with Ca^{2+} -binding proteins. Some of the potential applications include:

- 5 i) Identification of new Ca^{2+} -binding proteins. By applying protein containing sample to the chip; only Ca^{2+} -binding proteins would interact with the reagent bound to the chip. Those proteins could be eluted with a Ca^{2+} -containing buffer. Identification of the eluted proteins can be done by the techniques known in the art, such as MALDI-TOF, LC-MS/MS, interactions with antibodies, etc. The chip requires small amounts of the protein relative to column, and a Ca^{2+} -eluted protein can be obtained within 10 min.
- 10 ii) The second chip type allows identification of proteins interacting with Ca^{2+} -binding proteins. Proteins often function as a part of multi-protein compounds; identifying the individual proteins and determining their sites of interaction within the compound are essential for defining their mode of action and function. Chips containing known Ca^{2+} -binding proteins can be used for the identification of other proteins specifically interacting with them.

One of the challenges, facing life sciences researchers today, is bridging the gap between the knowledge of genomic sequence and its protein products at one side, and the understanding of the protein function and cellular behavior at the other side. This invention can contribute to said bridging. A novel chip containing several dozens of known Ca^{2+} -binding proteins can be used in searching for their interacting partners. Binding affinity and kinetics, binding stoichiometry, binding specificity, and epitope mapping can be obtained by charging the chip with known Ca^{2+} -binding proteins and studying their interaction with known proteins. Alternatively, the protein-charged chip can be used to search for unknown interacting protein. In this case the chip will be exposed to potential protein candidates. To identify the interacting protein, the chip will be exposed to Ca^{2+} or EGTA to dissociate said interacting protein from the chip, and the protein will be subjected to analysis by known methods, such as MALDI-TOF, LC-MS/MS, etc. The

invention, in one of its aspects, can provide a tool for fast screening of proteins, for example proteins that are involved in cross-talk within the cell or proteins modified in certain pathological conditions, thus adding another general technique to those provided by genomics and proteomics.

5

Example 12

AzRu inhibition of apoptotic and necrotic cell death

Cellular Ca^{2+} signals are crucial in the control of most physiological processes including cell injury and programmed cell death (apoptosis). This regulation is mediated through Ca^{2+} interaction with a number of Ca^{2+} -dependent enzymes such as phospholipases, proteases, and nucleases. Mitochondria and ER play pivotal roles in regulating intracellular Ca^{2+} content and thereby involved in apoptotic cell death. Moreover, members of the Bcl-2 family, a group of proteins that plays important roles in apoptosis regulation, appear to differentially regulate intracellular Ca^{2+} level.

The U-937 human monocytic cell line was grown under an atmosphere of 95% air and 5% CO_2 in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were plated at a density of 5.4×10^4 cells/ cm^2 in 24-well plates and then incubated with or without AzRu for 18 hr before exposure to 1.25 μM staurosporine (STS) to induce apoptosis. Cell viability was analyzed 5 and 7 hrs after STS addition by staining with 100 $\mu\text{g}/\text{ml}$ acridine orange (AcOr) and 100 $\mu\text{g}/\text{ml}$ ethidium bromide (EtBr) in PBS. The cells were then visualized by fluorescence microscopy (Olympus IX51) and images were recorded on an Olympus DP70 camera, using an SWB filter.

The effect of AzRu on apoptotic cell death induced by exposure of U-937 cells to STS is shown in Figure 13. Exposure of the cells to STS for 5 or 7 hrs resulted in apoptotic cell death of about 42% and 76% of the cells, respectively, whereas only 6% of the STS non-exposed cells died. AzRu, added 18 h before exposure to STS, dramatically reduced STS-activated apoptotic

cell death by about 75% and 47% after 5 and 7 h of exposure to STS, respectively. AzRu had no effect on the viability of the cells that were not exposed to STS. In figure 13 A, U-937 cells were incubated without (a, c) or with AzRu (10 μ M) (b,d) for 18 hrs and thereafter were exposed to STS (1.25 μ M) (c and d). After five hours the cells were stained with acridine orange and ethidium bromide. Arrows indicate cells in early apoptotic state, represented as degraded nucleus, but with intact cell membrane (stained by acridine orange). Arrowheads indicate a late apoptotic state, as shown by the presence of a degraded nucleus (stained by both acridine orange and ethidium bromide). Scale bar, 20 μ m. In figure 13 B, quantitative analysis of apoptotic cell death in control cells and cells exposed to AzRu was assessed 5 and 7 hr after their exposure to STS (1.25 μ M) by ANOVA and t-test. $P < 0.001$ was considered statistically significant (***). Data are the means \pm SEM, $n = 3$. In each independent experiment, approximately 200 cells were counted for each treatment.

Example 13

Comparison of RuR, Ru360 and AzRu effects

The hexavalent RuR and the trivalent Ru360 have been employed in various studies as inhibitors of Ca^{2+} -dependent reactions. As shown here, even with no photoactivation, AzRu, in contrast to RuR and Ru360, was effective in inhibiting the SR Ca^{2+} -pump (Table 3). AzRu was also more effective than RuR or Ru360, with or without photoactivation, in inhibiting the Ca^{2+} -dependent binding of ryanodine to RyR (Table 3). This may open the way to inhibit other Ca^{2+} -dependent activities that are not sensitive to RuR, such as the plasma membrane Ca^{2+} -ATPase. In intact cells, RuR was shown to slowly cross the plasma membrane and to protect against cell death induced by various stimuli. AzRu protected against apoptotic cell death induced by STS (Fig. 13), suggesting that AzRu is able to enter into the cell. RuR was employed to improve mitochondrial production in ischemic reperfused pig myocardium, was employed in the nervous system to follow neuronal death progress and can act as an anti-oxidant in certain conditions. Considering the

potent activity of AzRu in RuR-insensitive reactions (see Table 3), its applications in various biological studies should be explored. Ca^{2+} accumulation in SR and mitochondria was assayed as described above in the absence and presence of different concentrations of RuR (0.05-100 μM), Ru360 (0.01-100 μM) or AzRu (0.1-100 μM). The concentration required for 50% inhibition (IC_{50}) was derived from the plot of Ca^{2+} accumulation activity or ryanodine binding as a function of the reagent concentration. The results are the mean \pm SEM of 3 to 5 experiments (Table 3).

10 **Table 3** Comparison of RuR, Ru360 and AzRu sensitivity of different Ca^{2+} -dependent activities.

Assay	IC_{50} , μM			
	RuR	Ru360	AzRu	AzRu (+UV)
Ca^{2+} accumulation in SR	a	a	4.91 ± 0.055	1.01 ± 0.095
Ca^{2+} accumulation in mitochondria	0.16 ± 0.03	0.067 ± 0.008	1.78 ± 0.12	b
Ryanodine binding in SR	7.2 ± 0.55	14.8 ± 2.8	2.9 ± 0.17	1.21 ± 0.06

a - No inhibition, but rather stimulation was obtained.

15 b - Data not presented because UV irradiation of mitochondria resulted in partial inhibition of electron transport, and thereby inhibition of the membrane potential-supported Ca^{2+} accumulation.

20 While this invention has been described in terms of some specific examples, modifications and variations are possible. It is therefore understood that within the scope of the appended claims, the invention may be realized otherwise than as specifically described.

CLAIMS

1. A photoreactive azido-ruthenium (AzRu) compound which binds to a Ca^{2+} -binding protein.
2. The compound of claim 1, wherein the molar ratio between ruthenium and azide in said compound is 2:1.
3. The compound of claim 1 or claim 2, comprising in its molecule ruthenium, azido group, and chlorine.
4. The compound of claim 3, wherein the molar ratio $\text{Ru}:\text{N}_3:\text{Cl}$ is 2:1:5.
5. The compound of any one of claims 2 to 4, further comprising bound water molecules.
6. The compound of any one of claims 1 to 5, in which one of the atoms is a radioactive isotope.
7. The compound of claim 6, wherein the isotope is ^{103}Ru .
8. The compound of any one of claims 1 to 7, which compound covalently binds to said Ca^{2+} -binding protein following photo-activation by UV irradiation.
9. The compound of claim 8, which binds to the Ca^{2+} -binding site of said Ca^{2+} -binding protein.
10. The compound of any one of claims 1 to 9, which specifically binds to said Ca^{2+} -binding protein, thereby inhibiting its Ca^{2+} -dependent activity.
11. The compound of any one of claims 1 to 10, wherein said Ca^{2+} -binding protein is selected from the group consisting of proteins involved in signal transduction, muscle contraction, neurotransmitter release, hormone secretion, cell motility, apoptosis, fertilization, cell proliferation, cell mitosis and in gene expression; proteins associated

with Ca^{2+} -transport, Ca^{2+} -pumps, and with the mitochondrial uniporter; channel protein VDAC; Ca^{2+} -release channel/ryanodine receptor; IP_3 receptor, proteins involved in Ca^{2+} -efflux in mitochondria; and soluble Ca^{2+} binding proteins regulating various cellular activities.

12. The compound of claim 10 or 11, wherein the inhibition of the Ca^{2+} -dependent activity of said Ca^{2+} -binding protein increases by photo-activation, compared to the inhibition without photo-activation.
13. A method of isolating a Ca^{2+} -binding protein from a source comprising the same, which method comprises the steps of:
 - i) providing a source comprising a Ca^{2+} -binding protein;
 - ii) reacting said source with an AzRu compound of any one of claims 1 to 12, optionally under photo-activation by UV irradiation, whereby said Ca^{2+} -binding protein is bound to said compound;
 - iii) isolating the material bound to said compound obtained in step (ii); and
 - iv) releasing the Ca^{2+} -binding protein from the product obtained in step (iii).
14. A method according to claim 13, wherein the Ca^{2+} -binding protein is isolated by affinity chromatography.
15. A method according to claim 14, wherein said AzRu compound is bound to particles of porous polymer that are packed in a column, and wherein any Ca^{2+} -binding protein is retained in said column, while other proteins are eluted.
16. A method according to claim 14 or 15, wherein said retained Ca^{2+} -binding proteins are released from the column by calcium ions.
17. A method according to claim 15 or 16, wherein said particles comprise agarose, cellulose, or dextran.

18. A method for characterizing the structure of a Ca^{2+} -binding protein, wherein said Ca^{2+} -binding protein has been isolated by a method according to any one of claims 13 to 17, further comprising a method selected from the group consisting of electrophoresis, autoradiography, liquid chromatography, MALDI-TOF analysis, LC-MS/MS, protein sequencing, and a sequence homology search.
19. A method for the preparation of an AzRu-comprising bio-sensor chip comprising the steps of:
 - i) providing an azido-ruthenium compound comprising in its molecule ruthenium, azido group, and chlorine at a molar ratio of 2:1:5;
 - ii) binding said compound to a polymer such as dextran and coupling it to a suitable support, preferably gold-plated surface to give a chip; and
 - iii) optionally stabilizing the resulting chip.
20. Use of the chip of claim 19 for the isolation of Ca^{2+} -binding proteins, comprising :
 - i) exposing said chip to a biological sample comprising a Ca^{2+} -binding protein for a time sufficient for binding of the protein with the support-bound ruthenium compound to occur; and
 - ii) washing said chip with a buffer comprising either calcium or EGTA.
21. Use of the chip of claim 20, comprising surface plasmon resonance.
22. A method of screening for a calcium-binding substances, preferably Ca^{2+} -binding proteins, comprising the steps of:

- i) providing test substances, preferably proteins;
 - ii) contacting said substances with a ruthenium compound as defined in any one of claims 1 to 12 under conditions which allow binding to occur, preferably under UV irradiation;
 - iii) isolating from the reaction of (ii) those substances that specifically bind to said ruthenium compound; and
 - iv) releasing the substances obtained in step (iii) from the ruthenium compound by suitable means.
23. The method of claim 22 further comprising the step of testing the substances obtained in step (iv) for their Ca^{2+} -dependent activity.
24. A method according to any one of claims 13 to 18, wherein said AzRu compound is labeled.
25. A method according to claim 24, wherein said labeling is radioactive labeling.
26. A process for preparing a photoreactive azido-ruthenium compound which binds to a Ca^{2+} -binding protein, wherein the molar ratio between ruthenium and azide in said compound is 2:1, comprising:
- i) reacting in the dark sodium azide with ruthenium (III) chloride in the presence of HCl;
 - ii) applying the reaction mixture of the previous step onto a chromatographic column selected from cation-exchanger or hydrophobic;
 - iii) collecting the fractions having high absorbance at 290 nm; and optionally
 - iv) drying the collected fractions, redissolving them, rechromatographing them, and optionally crystallizing said compound from methanol.
27. The process of claim 26, wherein the HCl has the concentration in the range of from 0.5 mol/l to 2 mol/l.

28. The process of claim 26, wherein said sodium azide and ruthenium chloride are reacted at about 100°C for about 2 to 4 hrs.
29. The process of claim 26, wherein the product of AzRu migrates as a single spot with Rf being about 0.9 during TLC on cellulose F plates, using 0.16 M ammonium formate, pH 8.5 and 20% methanol as the developer.
30. The process of claim 26, wherein said product is soluble in water, DMF and DMSO, less soluble in methanol, and insoluble in ethanol, ether, chloroform, ethyl acetate, n-butanol, and isopropyl alcohol.
31. The process of claim 26, wherein said product has an absorbance maximum at about 290 nm.
32. The process of claim 26, wherein said product has absorbance of about 15,000 at 290 nm in a water solution, in the concentration of 1.0 M.
33. Use of an AzRu compound of any one of claims 1 to 12 in diagnosing a disorder associated with a defect in the function of a Ca²⁺-binding protein in a subject, comprising:
 - i) providing a sample of said subject and a control sample of a normal subject;
 - ii) contacting said samples with an azido-ruthenium compound as defined in any one of claims 1 to 12 under conditions suitable for binding to occur, preferably under UV irradiation;
 - iii) isolating from the mixtures obtained in ii) ruthenium-bound substances; and
 - iv) comparing the said substances obtained in iii) for said sample with the substances obtained in step iii) for said control sample;whereby a difference between the substances obtained in said sample and said control sample indicates a possible disorder in Ca²⁺-binding protein in said patient.

34. Use of an AzRu compound of any one of claims 1 to 12 in the preparation of a medicament for treating a disorder associated with a defect in the function of a Ca^{2+} -binding protein.
35. A pharmaceutical composition containing an AzRu compound, or a solvate thereof, prepared by a process of any one of claims 26 to 32.
36. A pharmaceutical composition according to claim 35, further comprising a carrier, stabilizer, adjuvant, diluent, or excipient.
37. A pharmaceutical composition according to claim 35, for use as a medicament for treating or preventing a disorder associated with a defect in the function of a Ca^{2+} -binding protein.
38. A pharmaceutical composition according to claim 37, for inhibiting the calcium-binding activity of said Ca^{2+} -binding protein.
39. A pharmaceutical composition according to claim 35, further inhibiting apoptotic or necrotic cell death.

1/11

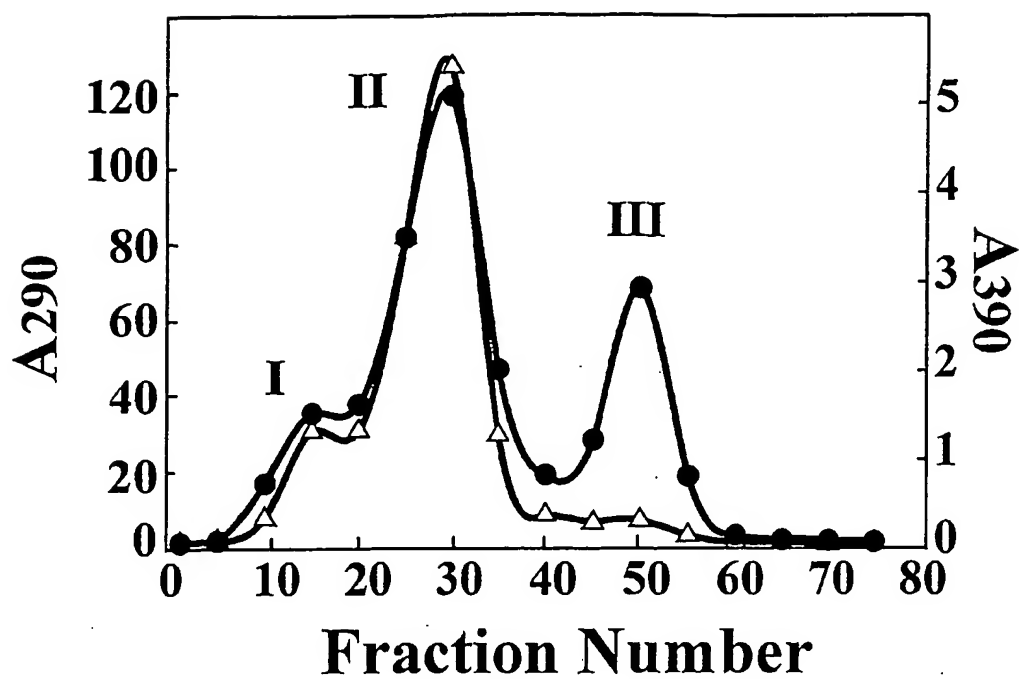


Fig. 1

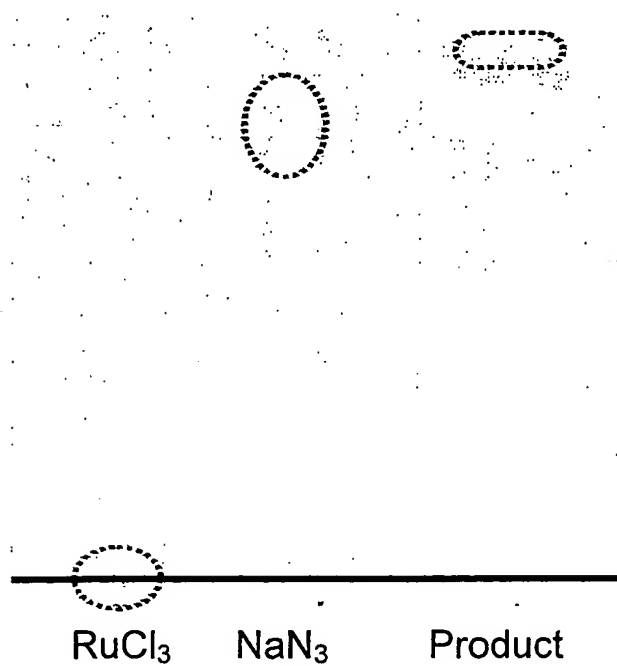


Fig. 2

2/11

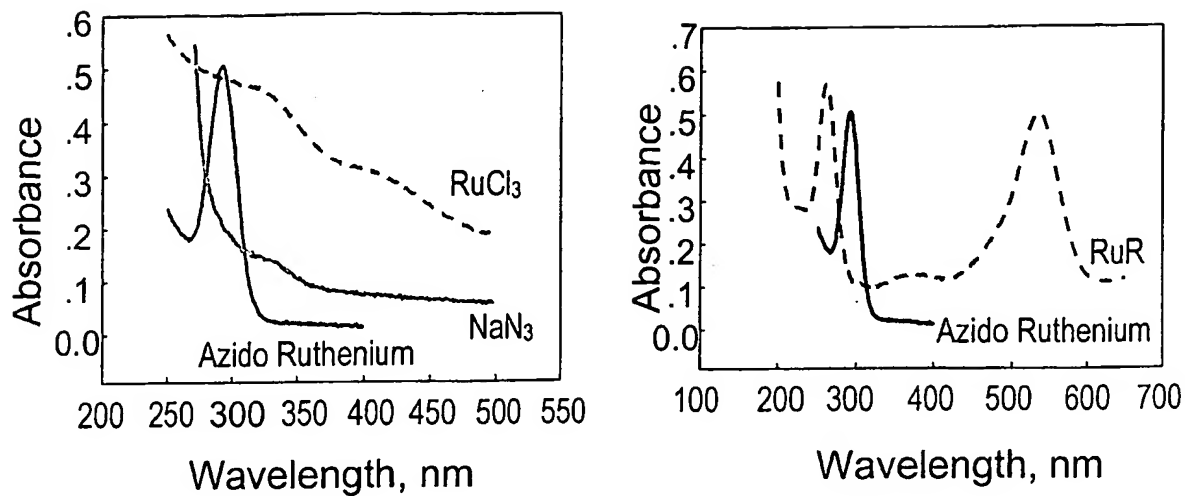


Fig. 3

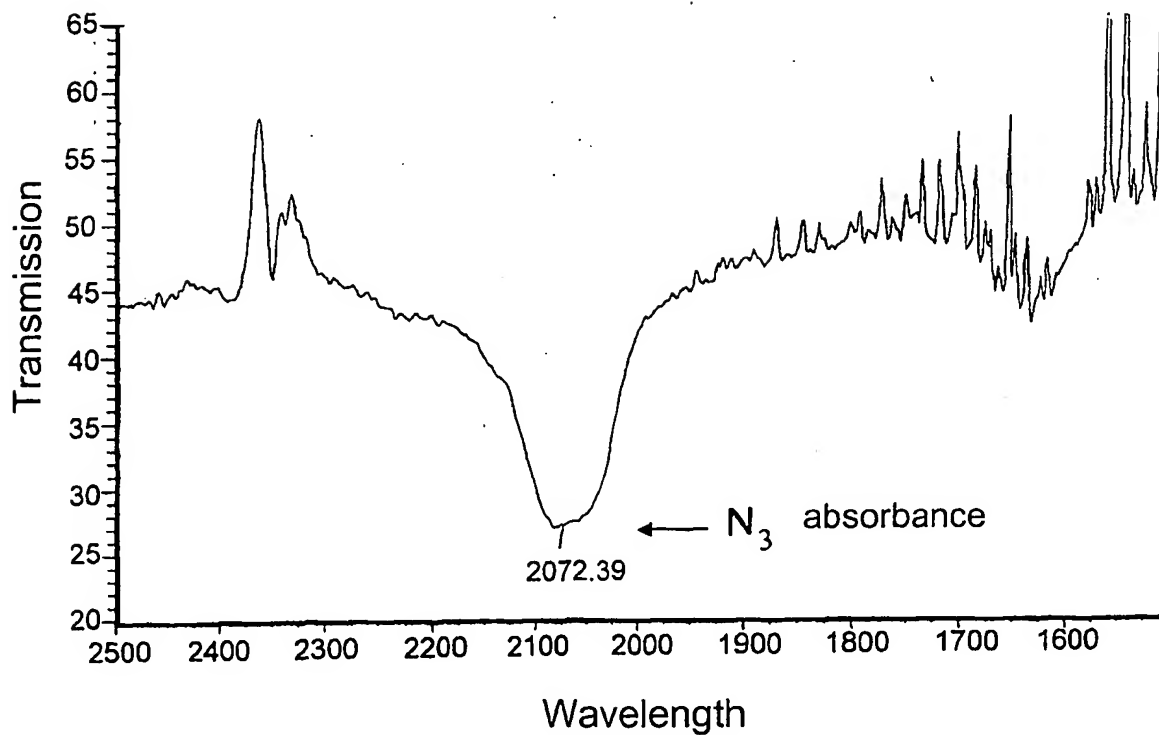


Fig. 4

3/11

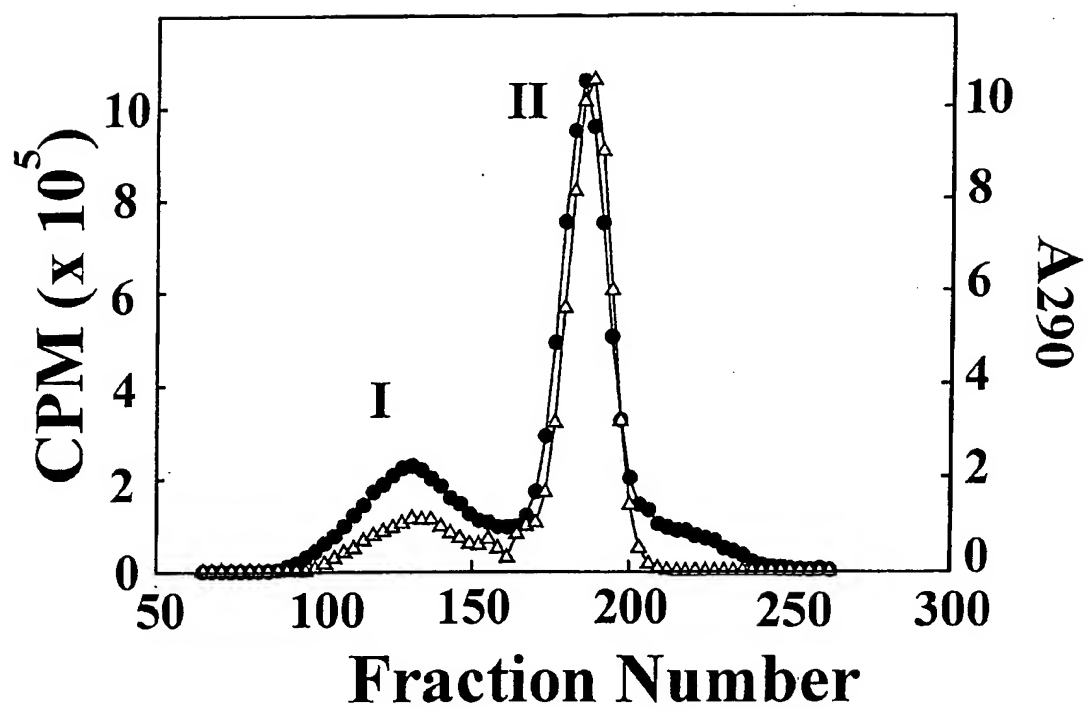


Fig. 5

4/11

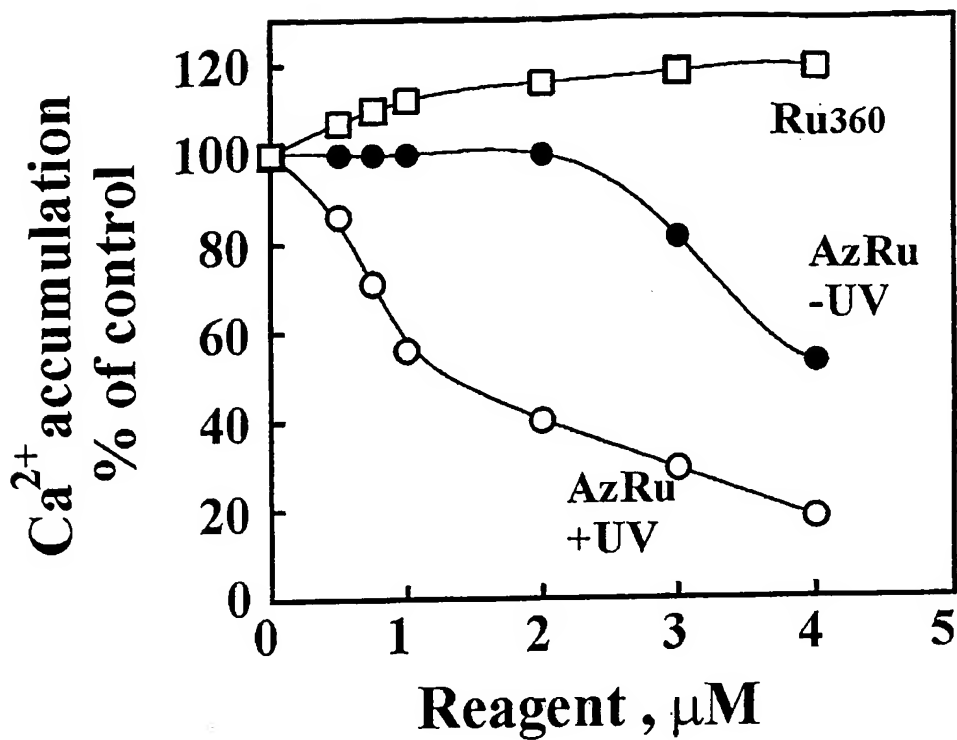


Fig. 6A

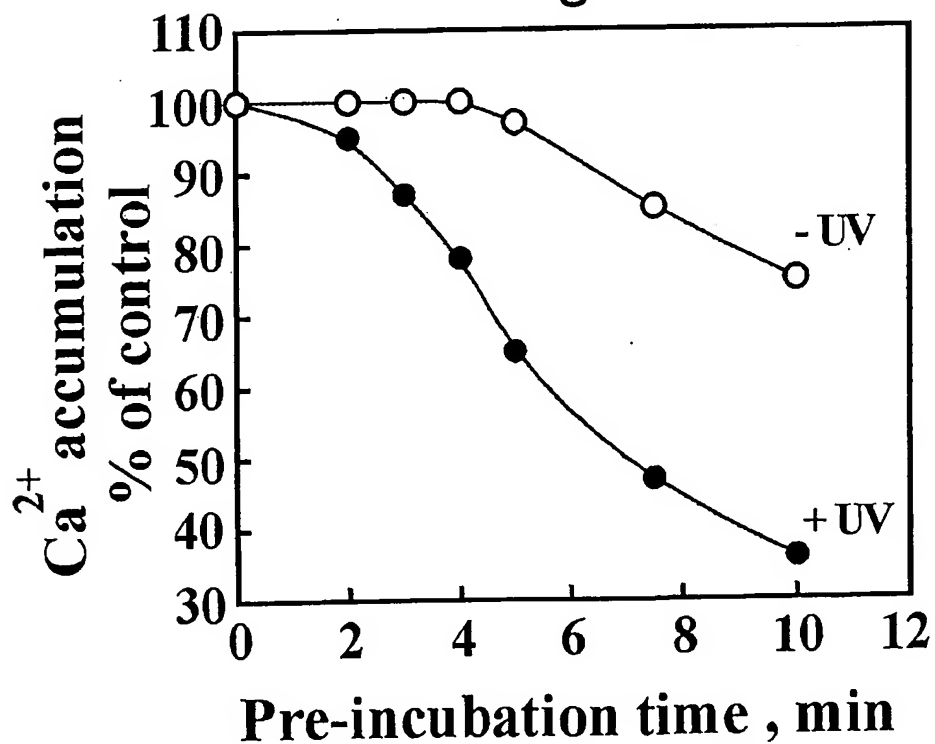


Fig. 6B

5/11

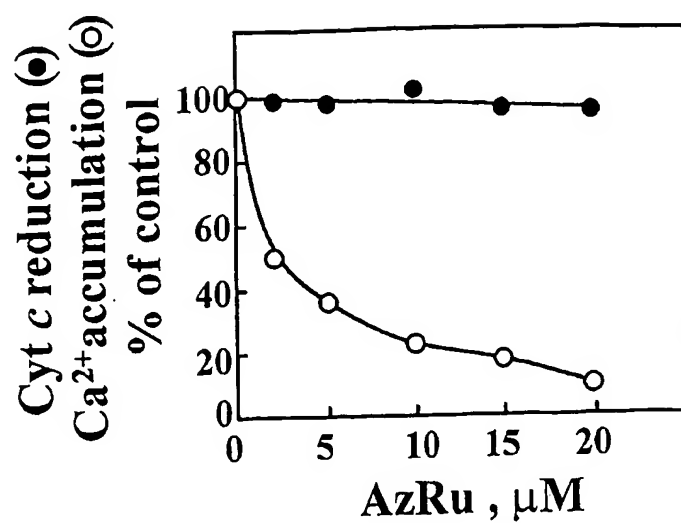


Fig. 7A

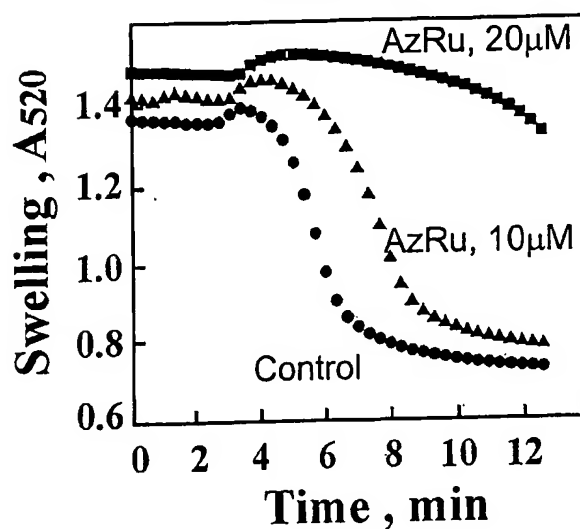


Fig. 7B

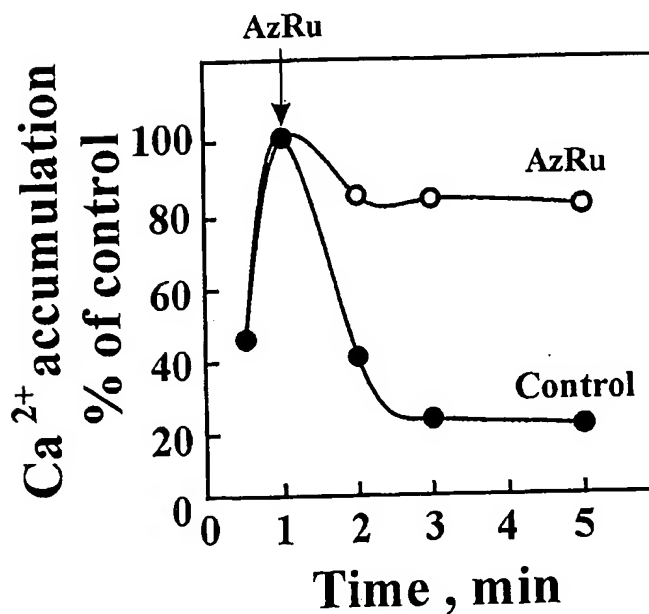


Fig. 7C

6/11

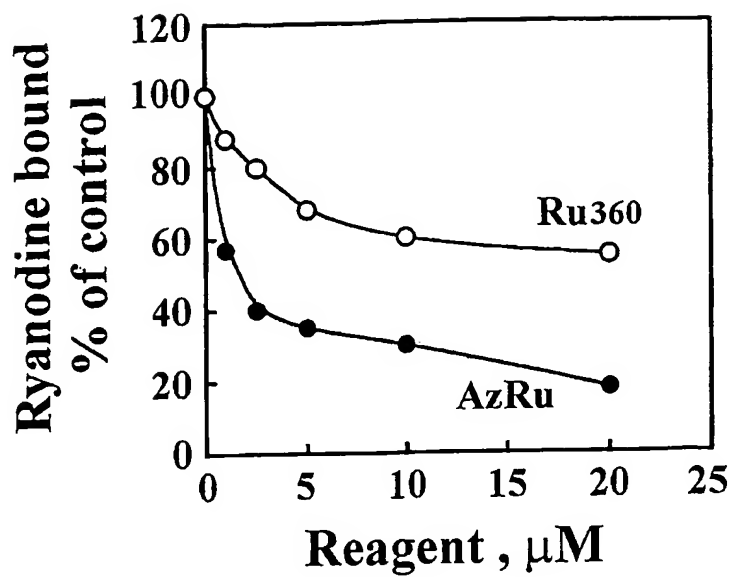


Fig. 8

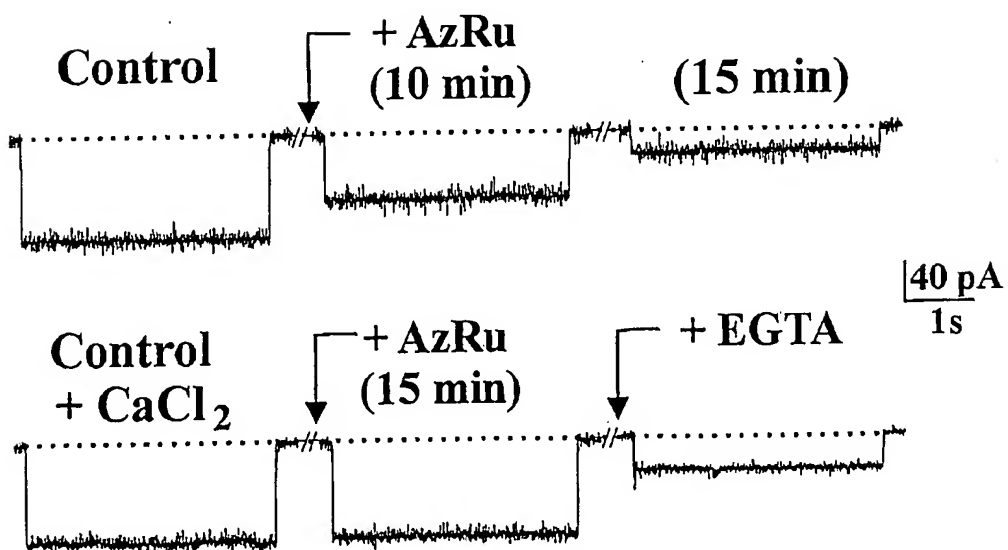


Fig. 9

7/11

Sarcoplasmic reticulum

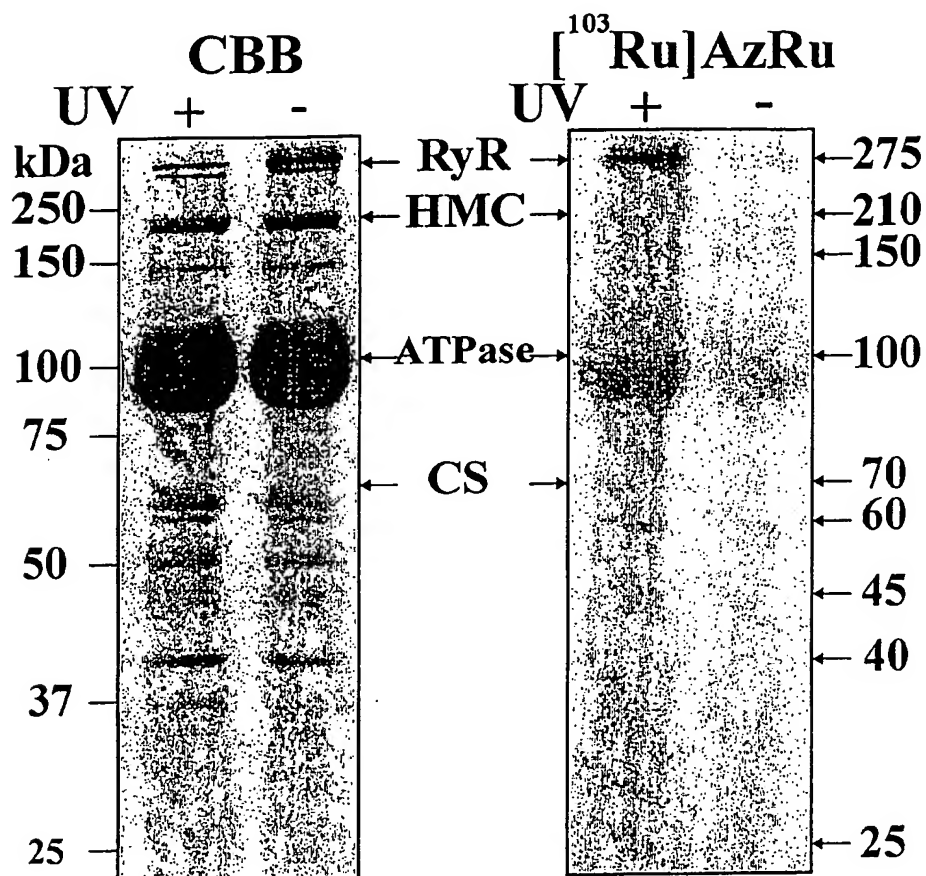


Fig. 10A

8/11

Mitochondria

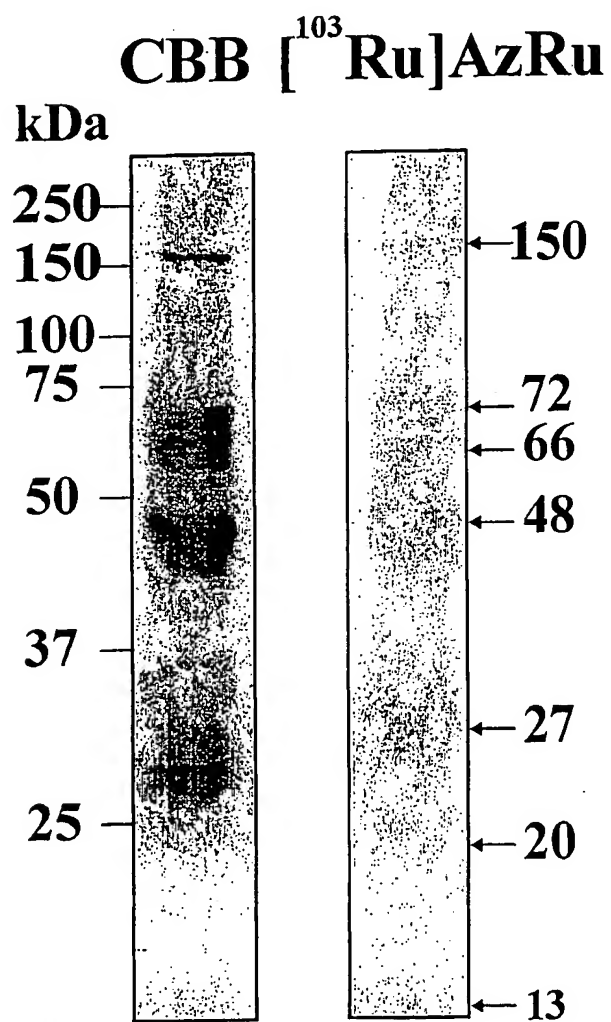


Fig. 10B

9/11

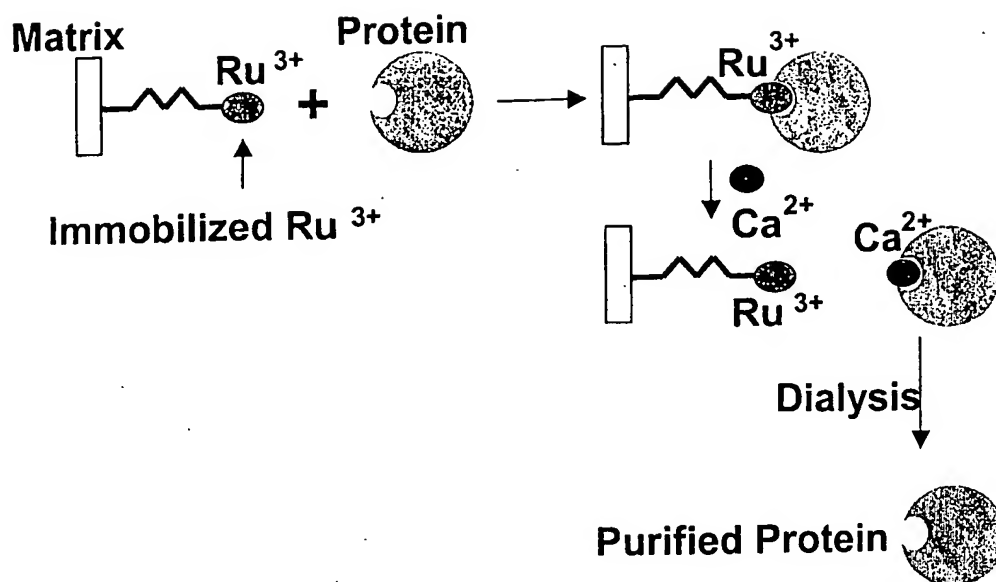


Fig. 11

10/11

Mito extracted
with:

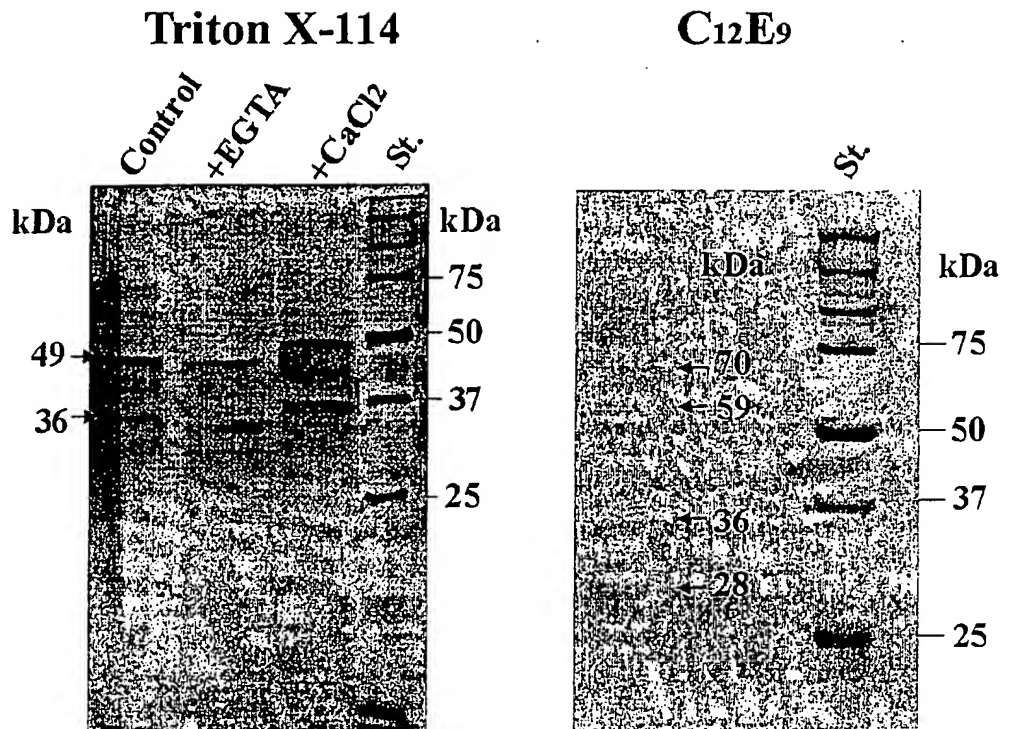


Fig. 12

11/11

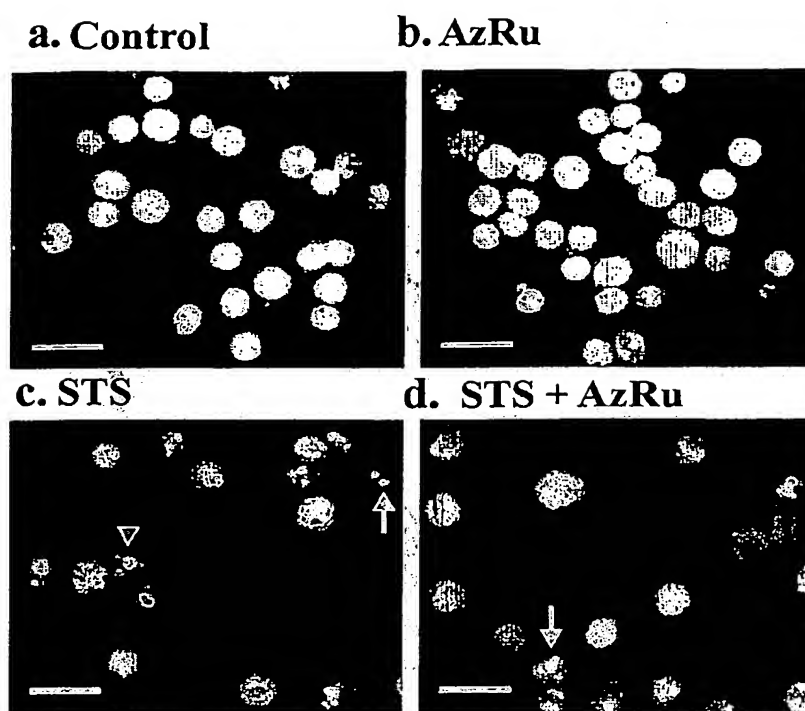


Fig. 13A

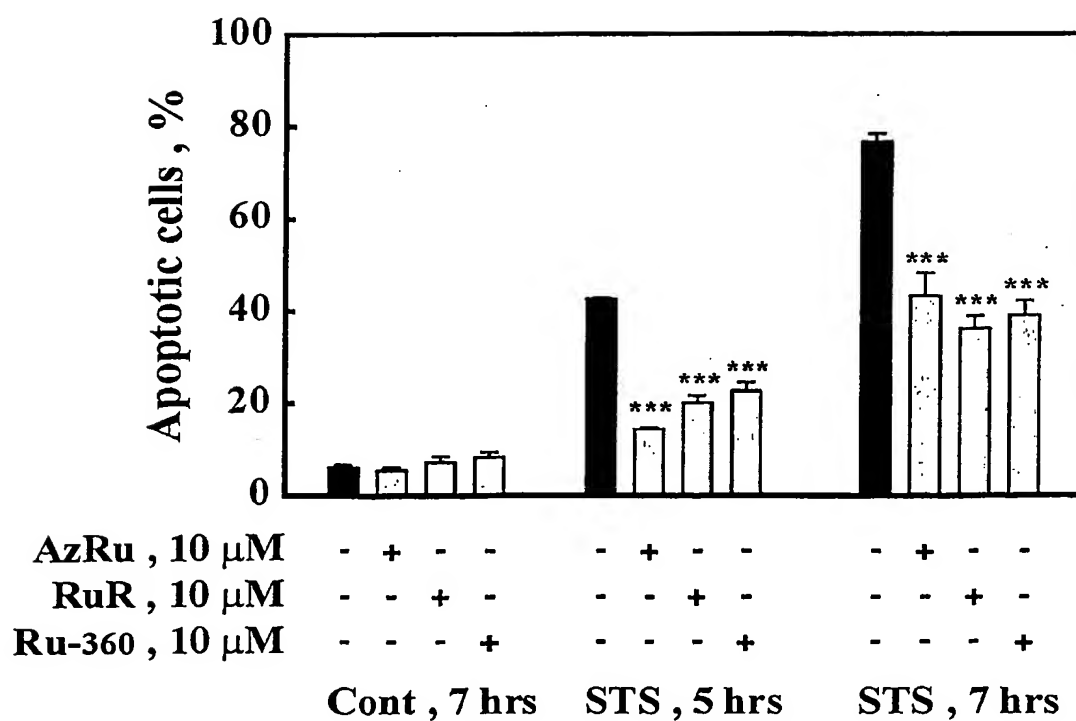


Fig. 13B

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record.**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.